APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1992, p. 2910–2917
0099-2240/92/092910-08$02.00/0

Vol. 58, No. 9

Isolation and Characterization of a Fluorene-Degrading Bacterium: Identification of Ring Oxidation and Ring Fission Products

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Received 26 December 1991/Accepted 22 June 1992

An *Arthrobacter* sp. strain, F101, able to use fluorene as the sole source of carbon and energy, was isolated from sludge from an oil refinery wastewater treatment plant. During growth in the presence of fluorene, four major metabolites were detected and isolated by thin-layer chromatography and high-performance liquid chromatography. 9-Fluorenol, 9*H*-fluoren-9-one, and 3,4-dihydrocoumarin were identified by UV spectra, mass spectrometry, and 300-MHz proton nuclear magnetic resonance. The fourth metabolite has been characterized, but precise identification was not possible. Since strain F101 is not able to grow with fluorenone, two different pathways of fluorene biodegradation are suggested: one supports cell growth and produces 3,4-dihydrocoumarin as an intermediate and probably the unidentified metabolite, and the other produces 9-fluorenol and 9*H*-fluoren-9-one and appears to be a dead-end route.

Polycyclic aromatic hydrocarbons (PAHs) are a class of fused-ring aromatic compounds which occur as ubiquitous environmental pollutants. Besides being present in petroleum, PAHs are formed during the incomplete combustion of almost any organic material (32), and they have been isolated from air (27), water (4), and river and marine sediments (16).

These compounds are of great environmental and human health concern because of their potential trophic biomagnification and because some low-molecular-weight PAHs are acutely toxic (7), and most higher-molecular-weight compounds have mutagenic, teratogenic, and potential carcinogenic effects (19, 30).

Fluorene, a tricyclic PAH which contains a five-membered ring, is a typical by-product of coal-conversion and energy-related industries and is commonly found in vehicle exhaust emissions, crude oils, motor oils, coal and oil combustion, waste incineration, and industrial effluents. Fluorene is one of the 16 PAHs on the list of priority pollutants compiled by the U.S. Environmental Protection Agency (21).

Microorganisms play an important role in the degradation of aromatic hydrocarbons in terrestrial and aquatic ecosystems. Microbial metabolism of lower-molecular-weight aromatic hydrocarbons, such as naphthalene, is well established (3, 15). Biodegradation of tricyclic compounds such as phenanthrene and anthracene has also been described (11, 17). In contrast, there is a relative paucity of information on the bacterial metabolism of compounds which contain a five-membered ring fused with aromatic rings such as indene, fluorene, acenaphthene, and fluoranthene. Schocken and Gibson (34) described the cooxidation of acenaphthene by a *Beijerinckia* sp. strain, and recent studies have reported the complete degradation of fluoranthene by pure cultures of *Pseudomonas paucimobilis* (31) and *Alcaligenes denitrificans* (38) and have suggested some of the details of the pathway. Weissenfels et al. (39) have proposed a pathway for the biodegradation of fluoranthene by *A. denitrificans* WW1 after the isolation and identification of two ring fission metabolites, acenaphthenone and 3-hydroxymethyl-3,4-benzocoumarin, and Kelley et al. have reported the mineralization of fluoranthene (22) and identified a carboxylic acid metabolite from the ring fission catabolism of this PAH by a *Mycobacterium* sp. strain (23).

Microbial degradation of fluorene at a hazardous-waste site has been reported (24). George and Neufeld (14) have described the fungal transformation in soil by a *Phanerochaete* sp., and Engesser et al. (10) have described the cometabolism of fluorene by dibenzofuran-grown cells. Recently, Weissenfels et al. (38) isolated a strain of *Pseudomonas vesicularis* which can utilize fluorene as the sole source of carbon and energy, but to our knowledge, no metabolites from the bacterial degradation of fluorene as the sole source of carbon and energy have yet been described. In this article, we report the isolation and characterization of an *Arthrobacter* sp. strain which utilizes fluorene as the sole source of carbon and energy and the identification of oxidation and ring fission products.

MATERIALS AND METHODS

Chemicals. Fluorene was purchased from Fluka AG, Buchs, Switzerland. 9-Fluorenol, 9*H*-fluoren-9-one, and 3,4-dihydrocoumarin were obtained from Aldrich Chemie, Steinheim, Germany. Bacterial media and reagents used to culture the *Arthrobacter* sp. strain were purchased from Oxoid Ltd., Basingstoke, Hampshire, England. Solvents for high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) were purchased from J. T. Baker B.V., Deventer, Holland. Solvents and other chemicals and reagents were of the highest purity available.

Enrichment cultures. Strain F101 was isolated from fluorene enrichment cultures inoculated with a preexisting phenanthrene-degrading mixed culture. This phenanthrene-degrading mixed culture had been obtained from enrichment cultures inoculated with a sludge sample from an oil refinery wastewater treatment plant.

The mineral salts medium (MSM) used as the basal

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medium contained (per liter of deionized water) 2 g of Na_2SO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.65 g of K_2HPO_4, 1 g of $(NH_4)Cl$, 2 g of KNO_3, and 0.01 g of $FeSO_4$ (12). The final pH of the medium was adjusted to 7.4 with 1 N HCl, and the medium was sterilized (121°C for 20 min) prior to the addition of the hydrocarbon substrate. This medium was supplemented, where indicated, with yeast extract or with vitamin B_{12} (the latter being designated MSM-B_{12}). The hydrocarbons phenanthrene and fluorene were supplied at 0.01% as a solution in acetone.

The phenanthrene-degrading mixed culture had been obtained as follows. Fifty milliliters of medium with 0.01% phenanthrene in 250-ml Erlenmeyer flasks was inoculated with 5 ml of sludge. Flasks were shaken (200 cycles per min) in the dark at 22°C. After 7 days of incubation, a 5.0-ml sample was diluted 1:10 (vol/vol) in fresh medium and incubated under the same conditions. Subsequent identical transfers were performed every 7 days. HPLC analysis showed that the microbial community obtained after the fourth transfer was capable of degrading 80% of the phenanthrene in 6 days.

A similar sequential enrichment process was performed with fluorene as the source of carbon and energy. An aliquot of the fourth transfer of the phenanthrene enrichment culture was used as the inoculum. After two transfers, a bright yellow color appeared in the medium which persisted until the next subculture. After three more successive transfers and after verification of fluorene utilization by HPLC, samples were serially diluted, plated on fluorene agar, and incubated for 10 days.

Solid isolation medium consisted of enrichment medium plus 1 g of yeast extract per liter solidified with 1.5% agar. After inoculation, the surfaces of the agar plates were sprayed with a 2% solution of fluorene in acetone. This treatment gave a visible uniform surface coat of hydrocarbon on the agar plates. Although some heterotrophic bacteria were able to grow in this medium, colonies surrounded by yellow zones were distinguished. This phenomenon, possibly caused by fluorene utilization, was used as a screening criterion of presumptive fluorene-degrading bacteria. These representative colonies were aseptically removed and subcultured. Bacterial growth and degradation of fluorene were determined as described below.

Maintenance, identification, and characterization of a fluorene-degrading microorganism. The fluorene-degrading isolate F101 was routinely maintained on the isolation medium (MSM with fluorene and 1 g of yeast extract per liter solidified with 1.5% agar). After extensive subculturing, F101 maintained its growth rate and fluorene-degrading capacity.

To assay its capacity to use fluorene as the sole source of carbon and energy, the F101 strain was screened for growth factors. A requirement for vitamin B_{12} was found. The ability of F101 to utilize another PAH as the sole source of carbon and energy for growth was also tested. The compounds were added to the liquid medium as crystals (anthracene) or in acetone solution (naphthalene, phenanthrene, and fluoranthene) at a final concentration of 0.01%. The medium was supplemented with vitamin B_{12} (40 μg/liter) or with yeast extract (250 mg/liter). Growth was estimated by bacterial protein concentration by the method of Lowry et al. (26) after 20 days of incubation at 22°C.

The cells were Gram and acid-fast stained. Bacterial samples for scanning electron microscopy were prepared by the methods of Gabriel (13). Micrographs were obtained with a Hitachi H.800 scanning electron microscope.

Since the cell morphology and distribution were indicative of the coryneform group and because the rod-coccus cycle presented was representative of a group of interrelated genera, it was necessary to analyze the amino acid composition of the cell wall. The material was prepared by the Aberdeen method (2), and the hydrolysate was analyzed with an LKB-Alpha-plus (Pharmacia) amino acid analyzer.

meta cleavage of catechol was evidenced by the method suggested by Hosokawa in Stanier et al. (37) in fluorene-grown cells. The bacterial strain obtained was identified by the morphological characteristics and selected standard physiological and biochemical tests listed in Table 1 (6, 36). Growth tests were carried out by inoculating strain F101 in test tubes containing MSM-B_{12} and the standard substrate.

Verification of fluorene utilization and culture conditions for metabolite isolation. Growth at the expense of fluorene was established by demonstrating an increase in bacterial protein, a decrease in concentration of fluorene, and formation of metabolites. Fluorene-grown cells from the late-exponential-growth phase were used as inoculum (5 μg of protein per ml). Replicate batch cultures were grown in 100-ml Erlenmeyer flasks containing 20 ml of MSM supplemented with 40 μg of vitamin B_{12} per liter and 0.01% fluorene. Incubation was performed at 22°C, with agitation at 200 rpm. Inoculated flasks without the carbon source and killed-cell flasks with fluorene were included as controls. At each sampling period over 5 days, replicated cultures and respective controls were removed. The protein concentration was measured by the method of Lowry et al. (26) for the entire contents of one experimental flask and one control flask. To assess fluorene disappearance and the formation of metabolites, other entire replicated cultures and controls were extracted and analyzed by HPLC.

To isolate and identify fluorene metabolites, serial biodegradation experiments were carried out in 1,000-ml Erlenmeyer flasks containing 250 ml of the same medium with 0.01% fluorene. In accordance with time course experiments, cultures were incubated for 4 days and extracted.

Chemical analysis and identification of metabolites. Metabolites and residual fluorene were extracted three times with 2 volumes of ethyl acetate after acidification to pH 2.5 with 0.1 N HCl. After drying with anhydrous Na_2SO_4, extracts were concentrated under reduced pressure at 30°C and evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in a small amount of methanol.

HPLC was performed on a Kontron model 620LC chromatograph (Kontron AG, Zurich, Switzerland) equipped with an Applied Biosystems 1000S diode array detector and a 5-μm C_{18} Chromspher column (25 cm by 4.6 mm [inside diameter]; Chrompack, Middelburg, Holland). Separation was achieved with either of the following methanol-water linear gradient systems: 50 to 95% (vol/vol) methanol in 30 min or 0 to 100% (vol/vol) methanol in 20 min.

To obtain fractions enriched in polar metabolites, preparative TLC was performed previous to HPLC. TLC was carried out by using precoated 500-μm-Silica Gel 60 DC plates (20 by 20 cm) (Merck). A hexane-acetone (8:2, vol/vol) solvent system was used to separate fluorene and nonpolar metabolites from polar metabolites, and a benzene-acetone-acetic acid (85:15:5, vol/vol/vol) solvent system was used for further separation of bands enriched in polar metabolites. Several bands were isolated and extracted with methanol in an ultrasonic bath. Only two of these bands enriched in polar metabolites (R_f, 0.30 and 0.40) showed an HPLC elution profile with a major metabolite peak, and these were further purified by multiple injections into an

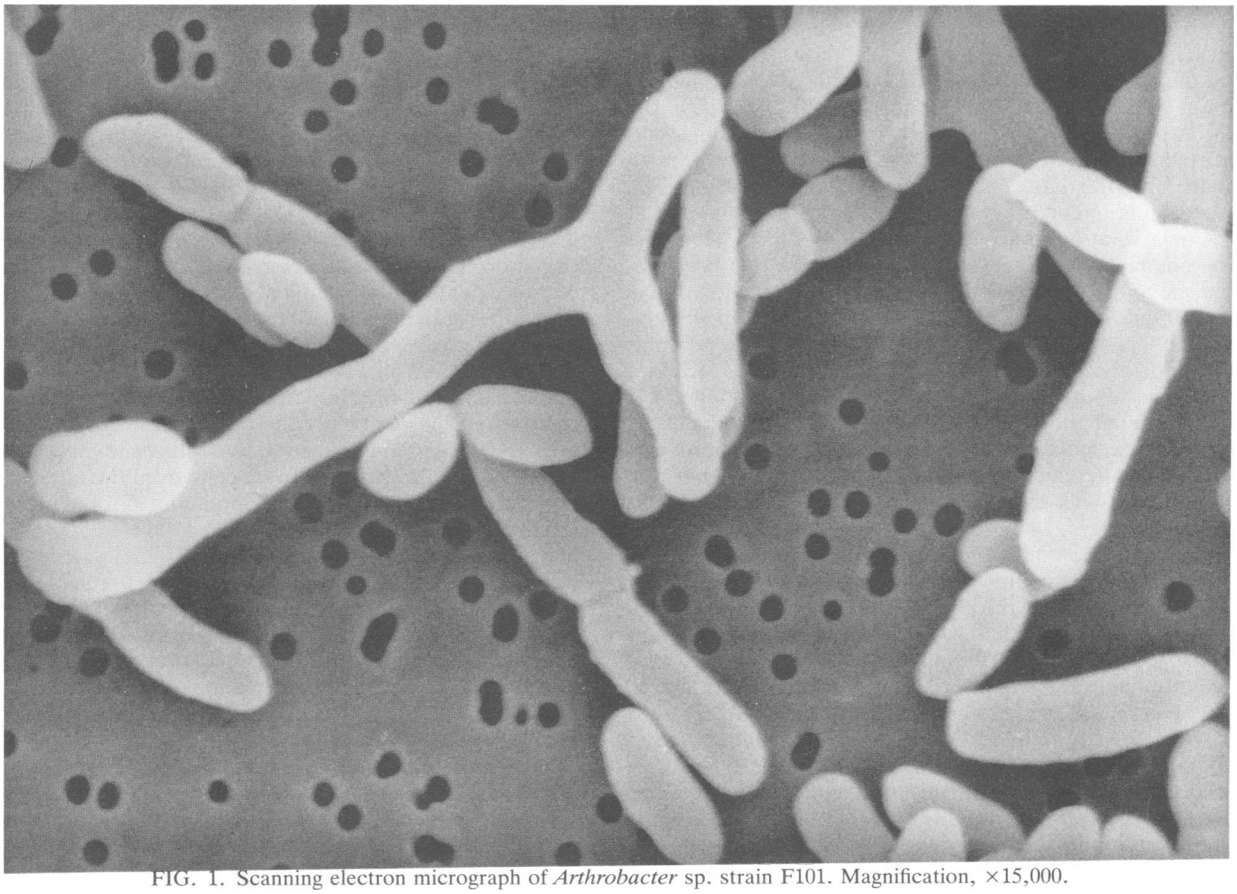

FIG. 1. Scanning electron micrograph of *Arthrobacter* sp. strain F101. Magnification, ×15,000.

HPLC. The subsequent collection of these metabolite peaks resulted in the accumulation of small amounts (0.5 to 1.8 mg) of purified metabolites.

The UV-visible absorption spectra of fluorene metabolites were determined in a diode array detector. Gas chromatography-mass spectrometry (GC-MS) analysis of the acid-extractable compounds of interest was accomplished by derivatization with BSFFA [*N*,*O*-bis(trimethylsilyl)trifluoroacetamide]. Infrared spectra of fluorene metabolites were determined on a Bomem model MB120 FT-IR spectrometer (Bomem, Quebec, Canada).

The isolated fluorene metabolites were analyzed by electron impact (EI) mass spectrometry, by using a direct introduction probe, and by GC-MS by using a Hewlett-Packard 5985A quadrupole interfaced to a 9825A data system.

The mass spectrometer was operated at 70 eV of electron ionization energy, and injector, transfer line, ion source, and analyzer temperatures were held at 250, 280, 180, and 120°C, respectively. In the direct introduction probe EI mass spectrometry analysis, a platinum wire probe was used and the temperature was programmed from 50 to 200°C at 40°C/min. In the GC-MS analysis, we used a capillary column (30 m by 0.25 mm [inside diameter]) coated with 0.25 μm of DB-5 and helium as the carrier gas (linear velocity, 25 cm/s). The column temperature was held isothermally at 50°C for 1 min and then programmed to 250°C at a rate of 8°C/min. All analyses were performed with the samples dissolved in ethyl acetate.

The ^{1}H-nuclear magnetic resonance spectra were recorded in a Unity-300 spectrometer (Varian, Sunnyvale, Calif.) of 300 MHz in CD_3OD (99.99%) at 1 mg/ml. A total of 1,024 scans were acquired in 100 min at 0.25 Hz of resolution (digital resolution, 0.05 Hz). The delay between acquisitions was 4 s. Chemical shifts are reported in parts per million downfield from the internal standard tetramethylsilane.

RESULTS

Isolation and characterization of a fluorene-degrading bacterium. Subculturing a previous phenanthrene-degrading mixed culture in MSM containing fluorene resulted in a microbial community enriched in fluorene degraders. After the second transfer, a yellow color appeared in the supernatant of the enrichment cultures, and HPLC analysis of 5-day cultures of the last transfer revealed the disappearance of 78% of the fluorene with respect to controls.

After plating and subculturing, we obtained an isolate, F101, which produced colonies with surrounding yellow zones on fluorene agar plates. Culturing of this strain in liquid medium in the presence of fluorene resulted in the rapid appearance of this color in the supernatant, which presented a maximum at 398 nm in UV-visible spectra, and this coloration persisted in time with slight variations. The appearance of yellow culture fluids often occurs during the degradation of aromatic compounds caused by the accumulation of *meta* cleavage products (35). Similar coloration of

TABLE 1. Physical and biochemical characteristics of *Arthrobacter* sp. strain F101

Characteristic	Results
Bacteriological tests	
Motility	−
Gram reaction	+
Morphology	Rod-coccus cycle present, irregular rods with angular arrangement, primary branching
Growth tests	
D-Glucose	+ (not acid)
L-Arabinose	−
D-Mannose	−
N-Acetylglucosamine	−
Maltose	+
D-Gluconate	−
Caprate	−
Adipate	+/−
L-Malate	−
Citrate	−
Phenylacetic acid	−
Biochemical tests	
Growth factor	Vitamin B_{12}
Oxidase	−
Catalase	+
Nitrate reduction	+
Glucose fermentation	−
Arginine dihydrolase	−
Urease	−
Esculin hydrolysis	+
Gelatinase	−
β-Galactosidase	+
DNase	+

medium was described by Mueller et al. (31) when *P. paucimobilis* was grown with fluoranthene.

The isolated organism exhibited a clotty growth in MSM with 40 μg of vitamin B_{12} per liter and with fluorene as the sole source of carbon and energy. *meta* cleavage of the catecholic ring was demonstrated in fluorene-grown cells.

In the maintenance medium in the absence of fluorene, strain F101 formed cream-colored colonies with diameters of about 1 mm. Cells of strain F101 are gram positive, although they lose the stain easily, especially in old cultures. This strain was not acid fast, and no spores were detected. Microscopic observations of exponential-growth-phase cultures showed irregular rods with angular arrangement and primary branching. A scanning electron micrograph showing morphological characteristics of individual cells which are typical of the coryneform group is presented in Fig. 1. In addition, cells changed shape during the growth cycle, from irregular rods to coccal forms. The rod-coccus growth cycle is a distinctive feature of the genus *Arthrobacter*, but it also occurs in other genera such as *Brevibacterium* and in some members of the genus *Rhodococcus* (36). Amino acid analysis of the cell wall showed the presence of diaminopimelic acid as diamino acid in the peptidoglycan. Table 1 presents the results of a variety of bacteriological, biochemical, and growth tests for this organism.

On the basis of all of these data, this organism was tentatively classified as one of the species in the genus *Arthrobacter* that have been shown to contain diaminopimelic acid in the cell wall peptidoglycan (36) and was designated strain F101.

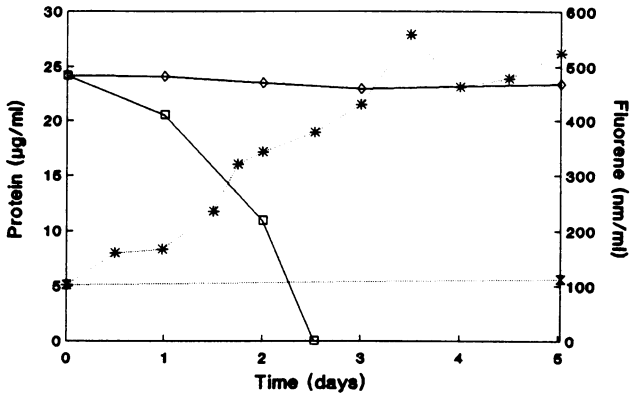

FIG. 2. Utilization of fluorene by *Arthrobacter* sp. strain F101 in liquid culture. Fluorene concentration with active (□) and heat-killed (◇) cells of strain F101 and biomass production by active cells of strain F101 with (*) and without (⧖) fluorene are shown.

Utilization of fluorene. Utilization of fluorene as the sole source of carbon and energy by strain F101 was demonstrated by its complete removal from MSM-B_{12} supplemented with fluorene, with a corresponding increase in bacterial biomass (Fig. 2) and appearance of metabolites. The concentration of fluorene decreased in 36 h from 483 nM/ml to levels not detected by HPLC analysis. Concomitantly, bacterial biomass increased from 5.09 to 27.87 μg/ml after 96 h of incubation. The corresponding generation time was 30.6 h. Nonsignificant change in the fluorene concentration was observed in heat-killed cultures. The slight decrease (483 to 444 nM/ml, over 5 days) in fluorene concentration seen in heat-killed cultures is attributed to loss by volatilization. The bacterial biomass remained unchanged in the absence of fluorene.

Of the PAHs assayed, phenanthrene was the only one other than fluorene that strain F101 was able to utilize as the sole source of carbon and energy. Strain F101 also seems able to transform fluoranthene, as evidenced by the appearance of a brown coloration in cultures and changes in the UV-visible spectrum of the supernatant with respect to that of controls when the medium is supplemented with 250 mg of yeast extract per liter.

Detection and identification of initial ring oxidation products and ring fission products. Earlier HPLC analysis of culture extracts showed the occurrence of two major metabolites and a group of unresolved highly polar compounds (Fig. 3). To isolate and identify the detected metabolites, serial biodegradation experiments were carried out.

The major metabolites, designated I and II, presented retention times of 14.8 and 18.4 min, respectively. The HPLC peak detected at 28.2 min was undegraded fluorene. No abiotic degradation of fluorene was detected in the control flask containing killed cells.

The UV absorption spectrum of metabolite I showed maxima at 210 and 280 nm and a shoulder at 230 nm (Fig. 3). The EI mass spectrum (see Fig. 5) exhibited a base peak at *m/z* 181, which was assigned as $(M-1)^+$, a characteristic of hydroaromatic compounds (25). Fragments at *m/z* 165 and 152 were assigned as $(M-OH)^+$ and $(M-CO_2H)^+$, respectively. The trimethylsilyl derivative of metabolite I exhibited a mass spectrum with a molecular ion of *m/z* 254, indicative of formation of a mono trimethylsilyl ether from a monohydroxy parent compound. Accordingly, a fluorenol isomer was postulated, which was finally assigned as 9-fluorenol on

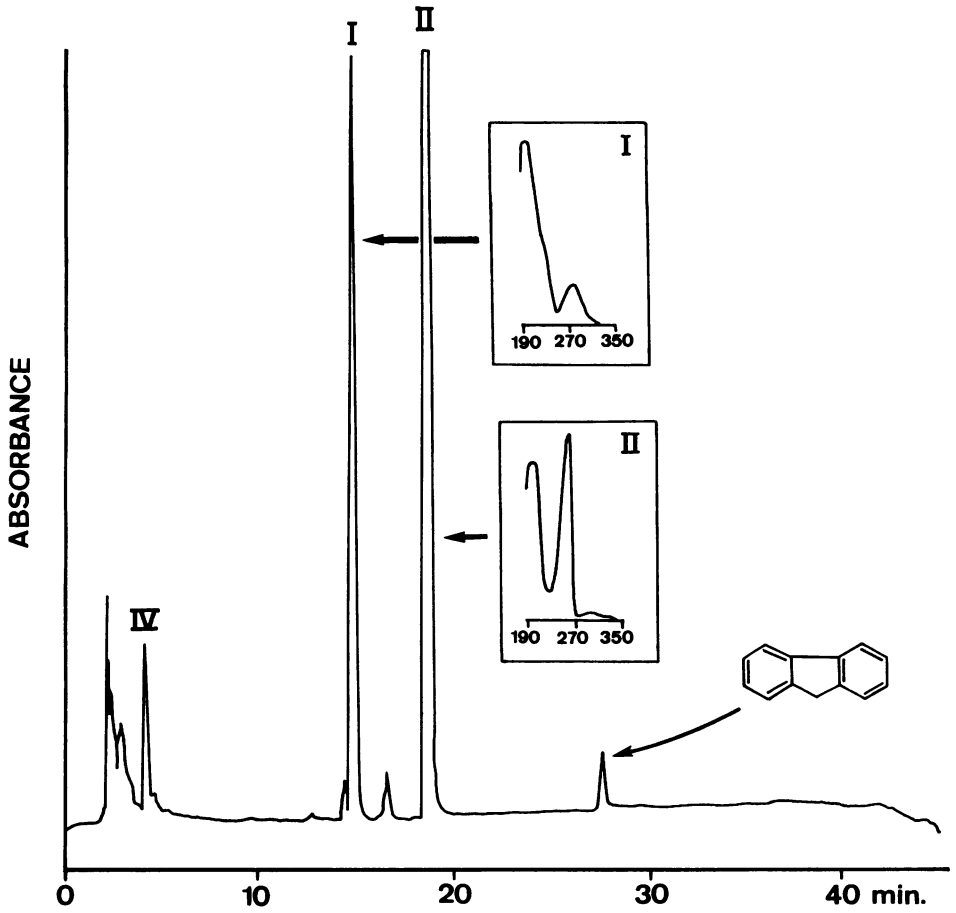

FIG. 3. HPLC elution profile showing the separation of metabolites I and II and a group of more-polar metabolites on a reversed-phase HPLC system with a methanol-water linear gradient system of 50 to 95% (vol/vol) methanol in 30 min. The UV spectra recorded in the maximum of each peak by the diode array detector are displayed as insets.

the basis of coinjection of authentic standards in capillary gas chromatography (CGC) analysis and HPLC and by comparison of EI and UV spectra.

The UV absorption spectrum of metabolite II showed maxima at 210 and 240 nm (Fig. 3). The molecular ion at *m/z* 180 was the base peak, and the fragment at *m/z* 152 was assigned as $(M\text{-}CO)^+$, suggesting a fluorenone structure (see Fig. 5). Final structural assignment was accomplished by coinjection of authentic standard in CGC and HPLC and by comparison of EI and UV spectra, and it was concluded that this metabolite was 9*H*-fluoren-9-one.

As mentioned above, a large amount of culture was required to purify and isolate the most-polar compounds. Enriched metabolite fractions from preparative TLC were obtained as described above. Two of the bands resolved were injected into an HPLC. Separation was achieved with a methanol-water linear gradient of 0 to 100% (vol/vol) methanol in 20 min. The HPLC profile of one of these bands showed a major metabolite peak, designated metabolite III (Fig. 4A), which was isolated by repeated injections into an HPLC and subsequent collection.

The UV absorption spectrum of metabolite III showed a maximum at 250 nm and a shoulder at 225 nm. The EI mass spectrum exhibited a molecular ion at *m/z* 148, which was the base peak. Fragments at *m/z* 120 $[(M\text{-}28)^+]$ and 91 $[(M\text{-}29\text{-}28)^+]$ were assigned as CO and HCO elimination. This mass spectrum matched that of 3,4-dihydrocoumarin found in a library search. Confirmation of its identity was obtained by comparison of properties with those of an authentic standard.

The other band resolved by TLC showed an HPLC elution profile (Fig. 4B) with several peaks. The main metabolic peak was designated metabolite IV. The UV absorption spectrum showed maxima at 210, 248, and 288 nm (Fig. 4B). The EI mass spectra (Fig. 5) exhibited a molecular ion at *m/z* 148, which was the base peak. On the basis of HPLC retention and UV and MS spectra, a polar-substituted aromatic structure is suggested.

Furthermore, ^{1}H-nuclear magnetic resonance analysis was performed for structural elucidation of this metabolite. The spectral assignments were as follows: (MeOH-*d*4) 2.52 (dd, 1, J_1 = 19 Hz, J_2 = 3 Hz), 3.1 (dd, 1, J_1 = 19 Hz, J_2 = 7.0 Hz), 5.37 (dd, 1, J_1 = 7 Hz, J_2 = 3 Hz), 7.52 (m, 1), 7.76–7.68 (Ca, 3) ppm. The lack of authentic standards prevented structural assignment. Current research is in progress to identify the metabolite by using high-resolution mass spectrometry.

The metabolites identified seem to suggest two pathways in the degradation of fluorene by *Arthrobacter* sp. strain F101: one pathway which supports the growth of the strain by *meta* cleavage of one of its aromatic rings, and the other

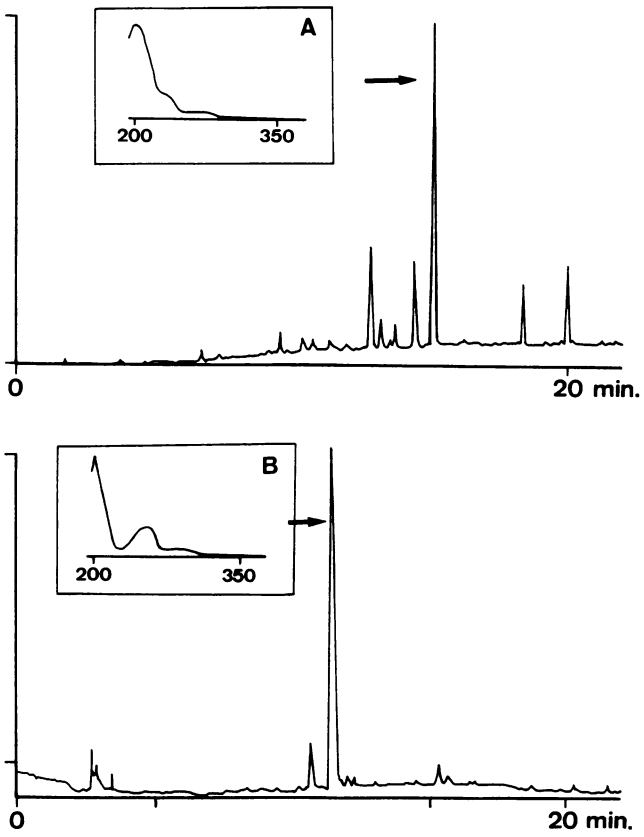

FIG. 4. HPLC elution profiles of two TLC-resolved bands corresponding to polar metabolites showing metabolite III identified as 3,4-dihydrocoumarin (A) and showing the unidentified fluorene metabolite IV (B). The separation was achieved with a methanol-water linear gradient system of 0 to 100% (vol/vol) methanol in 20 min. Insets, UV absorption spectra of the metabolites.

which led to formation and accumulation of 9*H*-fluoren-9-one via 9-fluorenol.

To verify that the latter pathway is a dead-end route in the biodegradation of fluorene by strain F101 and that this strain is able to oxidize 9-fluorenol to 9*H*-fluoren-9-one, incubations with these two products, at different concentrations (0.01, 0.001, and 0.005%), were carried out with the corresponding controls. No increase in biomass was observed in any of these incubations, and the HPLC profiles indicated that the strain was able to convert 9-fluorenol to 9*H*-fluoren-9-one, which accumulates, and that this conversion could be distinguished from a low rate of spontaneous oxidation seen in controls. Likewise, in the incubations of different concentrations of 9*H*-fluoren-9-one, no noticeable peak was observed.

DISCUSSION

Fluorene and its derivatives have been regularly found in environmental samples and identified in waters, soil, sediments, fish, and smoked foods (15, 20). This PAH is not genotoxic but has a chemical structure found in several mutagenic and/or carcinogenic PAHs such as 2-aminofluorene, 2-nitrofluorene, and 2-acetylaminofluorene (30).

In this article, we report the isolation of an *Arthrobacter* sp. strain which was able to grow with fluorene as the sole source of carbon and energy. This ability was demonstrated

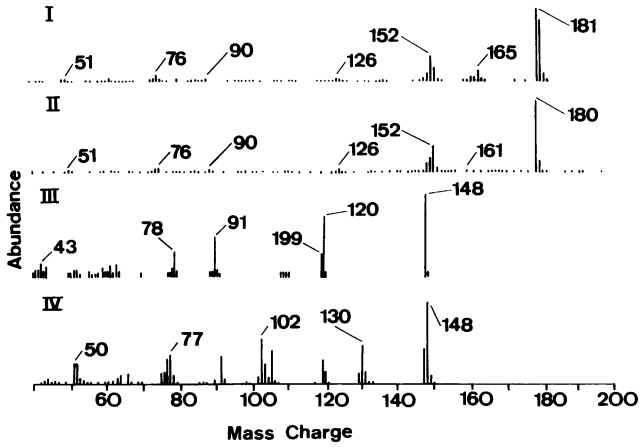

FIG. 5. EI mass spectra of metabolites I, II, III, and IV, identified as 9-fluorenol, 9*H*-fluoren-9-one, 3,4-dihydrocoumarin, and an unidentified metabolite, respectively, produced by *Arthrobacter* sp. strain F101 growing on fluorene.

by complete removal of fluorene in MSM-B_{12}, which led to a corresponding increase in bacterial biomass (Fig. 4). On the basis of morphological (Fig. 1), growth, and biochemical tests (Table 1), this organism was classified as a species of the genus *Arthrobacter*, a subgroup of which has been shown to contain diaminopimelic acid in the cell wall peptidoglycan (36).

During growth on fluorene in batch cultures, four metabolic products were detected. On the basis of UV, infrared, mass spectrometry, and nuclear magnetic resonance spectroscopy analyses, these compounds were identified as 9-fluorenol, 9*H*-fluoren-9-one, 3,4-dihydrocoumarin, and another polar metabolite of unknown structure (*m/z*, 148) which is presumed to have been formed by ring fission of one of the aromatic rings of fluorene.

Identification of these intermediates and the fact that biodegradation experiments in the presence of 9-fluorenol and 9*H*-fluoren-9-one did not show increase in bacterial biomass or the presence of further oxidative metabolites suggest that *Arthrobacter* sp. strain F101 has two different

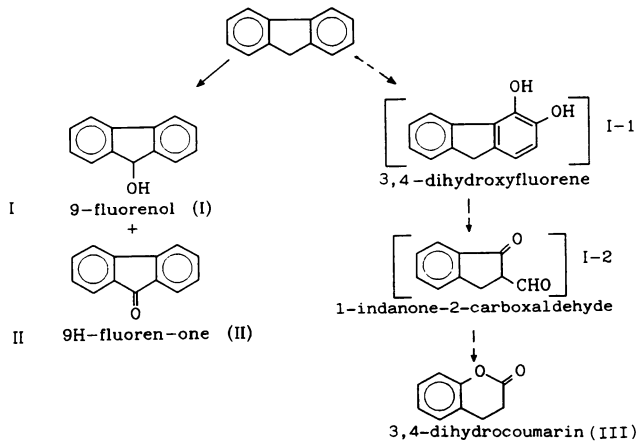

FIG. 6. Schematic pathway proposed for the degradation of fluorene by *Arthrobacter* sp. strain F101. The structures shown in brackets are proposed intermediates (I-1 and I-2) and have not been isolated.

pathways for the metabolism of fluorene (Fig. 6): one supports cell growth and produces 3,4-dihydrocoumarin as an intermediate, and probably the unidentified metabolite, and the other produces 9-fluorenol and 9*H*-fluoren-9-one and appears to be a dead-end route.

The initial oxidative step of the latter route would be the formation of the alcohol 9-fluorenol by monooxygenation. The second step would lead to the formation of the corresponding ketone, the 9*H*-fluoren-9-one, probably by dehydrogenation, which appears to be the end product of this pathway. Schocken and Gibson have described the same kind of reactions in the cooxidation of acenaphthene by a *Beijerinckia* sp. (34) to give 1-acenaphthenol, which is further dehydrogenated to 1-acenaphthenone by a dehydrogenase which also exhibits activity with 9-fluorenol. However, the presumed end product, 9*H*-fluoren-9-one, could be subjected to further degradation by other microorganisms in mixed microbial populations. Mormile and Atlas (28) reported that dibenzothiophene sulfone, the end product of a side reaction in the metabolism of dibenzothiophene (29) by *Pseudomonas putida*, can be degraded by mixed enrichment cultures. Indeed, George and Neufeld (14) have described the biodegradation of 9-*H*-fluoren-9-one by the fungus *Phanerochaete chrysosporium* in soils. Nevertheless, 9*H*-fluoren-9-one is a persistent contaminant, as evidenced by its widespread occurrence in polluted coastal sediments (16), and it experiences high ecological magnification, as has been demonstrated in model ecosystem studies (33).

The structure of metabolite III, 3,4-dihydrocoumarin, can only be obtained by complete degradation of the terminally exposed aromatic ring of fluorene. Considering the known pathways of degradation of similar PAHs, such as phenanthrene, anthracene, and fluoranthene, the *Arthrobacter* sp. attacks fluorene presumably via a dioxygenase at the 3,4 position to form the corresponding dihydrodiol, which undergoes enzymatic dehydrogenation to form 3,4-dihydroxyfluorene (Fig. 6, I-1). Further degradation could follow the general principles of PAH metabolism reviewed by Gibson and Subramanian (15).

A second dioxygenation would produce *meta* cleavage of the diol, and a further aldolase reaction on the corresponding ring fission product, similar to that described in the biodegradation of naphthalene (8), would give pyruvate and 1-indanone-2-carboxaldehyde (Fig. 6, I-2). The subsequent reaction would require an insertion of one oxygen atom into the carbon ring system by a Baeyer-Villiger reaction (1) after the mechanism of cleavage of alicyclic rings such as cyclohexanol (9) and camphor (5). Loss of the aldehyde substituent could occur by hydrolysis of the β-diketone or after oxidation to carboxyl and subsequent decarboxylation of the resulting β-keto acid. A similar mechanism may account for the compound acenaphthenone observed by Weissenfels et al. (39) in studies of fluoranthene biodegradation. Heitkamp et al. (18) also proposes a mechanism of 1-carbon excision in the formation of 4-phenanthroic acid detected in experiments of pyrene degradation by a *Mycobacterium* sp. strain.

These new aspects of the degradative pathway of fluorene by *Arthrobacter* sp. strain F101 introduce a new perspective to the study of biodegradation of naphthenoaromatic compounds, in which alicyclic and aromatic ring cleavage mechanisms are combined. Nevertheless, further research into the identification of other ring oxidation and ring fission products from F101 and other fluorene-degrading strains and the elucidation of the enzymatic activities involved are necessary to provide better knowledge of the bacterial metabolism of fluorene and its derivatives. Work along these lines is now in progress in our laboratory.

ACKNOWLEDGMENTS

Financial support was obtained from the National Plan for Research (grant NAT89-0927) and the Spanish Ministry of Public Works (MOPU). Two of us (M.G. and M.C.) were recipients of a Ph.D. fellowship from the Spanish Ministry of Education.

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